ANTIPREDATOR BEHAVIOR AND MORPHOLOGY IN ISOLATED CYPRINODONT FISHES

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

For desert fishes in the American Southwest, predation by invasive species has triggered massive population declines for decades, leaving researchers speculating on the underlying cause. It has been shown that Post-Pleistocene isolation of desert fishes in small habitats with limited predation pressure has led to loss of antipredator traits. Determining the status of antipredator behavioral and morphological traits could identify the most vulnerable desert fishes. In aquatic ecosystems, detection and response to chemical alarm cues derived from epithelial tissue increases the probability of predation survival. In chapter two, I evaluate alarm cue responses of two desert cyprinodontids: endangered Pahrump poolfish and Amargosa pupfish. In chapter three, I assess the prevalence and densities of epithelial club cells, the source of chemical alarm cues, for several desert fishes: Pahrump poolfish, Amargosa pupfish, White Sands pupfish, White River Springfish, and Hot Creek Valley tui chub.

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DEDICATION

This thesis is dedicated to:

The loving memory of my grandfather,

Christy L. Snider

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1. INTRODUCTION

1.1. Threats to Desert Fishes

Although known for an arid climate, the western deserts of North America have a unique array of natural aquatic habitats, ranging from freshwater springs to saline creeks with salinities up to three times that of the ocean (Soltz and Naiman 1978). Once connected by vast Pleistocene lakes, these small pools and ponds now rely on underground springs, mountain-fed streams, and rainfall to sustain aquatic life (Soltz and Naiman 1978). Aquatic ecosystems found in these waters are relatively simple, containing various aquatic invertebrates and only a few fish species (Minckley and Deacon 1968; Pister 1974; Minckley and Deacon 1991; Rundel and Gibson 1996). Because these communities often include only a few fish species, they have limited levels of predation and competition by less than a handful of fish predators and competitors (Miller 1948; Pister 1974; Soltz and Naiman 1978; Rundel and Gibson 1996).

Anthropogenic changes to the environment within the last century have led to numerous desert fishes being added to the endangered species list. A major and consistent threat driving these population declines has been the spread of invasive species. Declines and extinctions of many native fishes have been associated with the introduction of various non-native fishes such as largemouth bass (Micopterus salmoides) and the western mosquitofish (Gambusia affinis) (Miller 1961; Hubbs and Brodrick 1963; Deacon et al. 1964; Pister 1974; Meffe et al. 1983; Stockwell and Henkanaththegedara 2011). For example, extinction of the Monkey Springs pupfish (Cyprinodon arcuatus) coincided with the un-authorized introduction of largemouth bass (Minckley and Marsh 2009). Similarly, western mosquitofish introductions have been associated with the decline of numerous desert fishes (Minckley and Deacon 1968; Williams et al. 1985; Miller et al. 1989; Pyke 2008). This species, along with eastern mosquitofish (Gambusia

holbrooki), were deliberately introduced in different areas around the globe for mosquito control starting in the early 20th century (Pyke 2008). For instance, western mosquitofish were established in seven hatcheries through California in the early 1900s and subsequently introduced to Nevada in the 1930s (Stockwell et al. 1996).

1.2. Invasive Species Impacts

Able to colonize diverse habitats, western mosquitofish impact native fish populations through hybridization, resource competition, and aggressive predation on all life stages of other fishes (Pyke 2008; Stockwell and Henkanaththegedara 2011). For example, invasive mosquitofish were partly responsible for the range-wide decline of the Gila topminnow (Poeciliopsis occidentalis). Once considered the most abundant fish species in the Colorado River drainage, Gila topminnow now occupies a small fraction of its former range (Minckley and Deacon 1968; Sheffer et al. 1997). Similarly, population declines of White River spinedace (Lepidomeda albivallis) were observed soon after the introduction of western mosquitofish (Minckley and Marsh 2009). Invasive species have also caused fishes to change behaviors and habitats (Minckley and Deacon 1968). For instance, following the introduction of western mosquitofish, the least chub (Iotichthys phlegethontis) became nocturnal and moved to sub-optimal habitats (Ayala et al. 2007).

Continued presence of invasive species makes recovery of desert fishes challenging in the absence of substantial active management. For example, the establishment of refuge populations has been widely used for managing endangered fishes (Minckley and Deacon 1968; Soltz and Naiman 1978; Minckley 1995; Stockwell and Leberg 2002). Such refuge habitats typically are designed to host a single species (Miller 1948; Minckley and Deacon 1968; Minckley 1995; Goodchild and Stockwell 2016). However, habitats without fishes are at a premium, and removal

of invasive fish species is costly and often un-successful (Minckley 1995). Thus, re-evaluating constraints and exploring novel solutions for conservation solutions are both necessary to assist management procedures.

A historic constraint on management has been the pervasive view that non-native species always negatively impact desert fish populations (Stockwell and Henkanaththegedara 2011; Henkanaththegedara and Stockwell 2014). In turn, observed negative impacts have often been attributed to the evolutionary naïveté of desert fishes (Meffe 1985; Cox and Lima 2006). This view reflects long-held views concerning the vulnerability of naïve island species of terrestrial vertebrates. Reduced predation pressure is expected to lead to the loss of antipredator behaviors, such as high vigilance, hiding, and even predator avoidance (Roemer et al. 2002; Blumstein and Daniel 2005; Berger et al. 2007). The best documented examples of this phenomenon come from island populations of terrestrial vertebrates (Blumstein and Daniel 2005). For instance, antipredator behaviors such as flight initiation distances of marine iguanas (Amblyrhynchus cristatus) differ between islands of acute, low, or no predation in the Galápagos Islands (Berger et al. 2007). Populations lacking antipredator behaviors are said to be predatorily naïve (Roemer et al. 2002; Blumstein and Daniel 2005; Cox and Lima 2006; Berger et al. 2007).

1.3. Variation in Co-Persistence

While terrestrial island populations have been extensively studied, little empirical work has focused on fishes isolated in aquatic islands in the western deserts of North America. However, recent research on desert fishes suggested that interactions of invasive and native fishes may be more complicated than once thought. For example, Henkanaththegedara and Stockwell (2014) reported evidence that intra-guild predation may facilitate co-persistence of non-native western mosquitofish with the endangered Mohave tui chub (Siphateles bicolor

mohavensis). They found that mosquitofish predation on tui chub larvae was limited by gape size of the mosquitofish predator, allowing larger individuals to escape (Henkanaththegedara and Stockwell 2014). Reciprocally, adult tui chub predation on adult mosquitofish was also gape-limited. Similarly, Goodchild and Stockwell (2016) provided evidence that Pahrump poolfish could not persist in sympatry with mosquitofish or pupfish, whereas the Amargosa pupfish (Cyprinodon nevadensis) could co-persist with mosquitofish and/or native Pahrump poolfish (Empetrichthys latos). By contrast, Rogowski and Stockwell (2006) reported that invasive mosquitofish impacted population growth of experimental populations of the White Sands pupfish (C. tularosa). Thus, the impacts of introduced mosquitofish on native fishes appear to vary among desert aquatic systems, despite the fact that all of these systems are relatively simple. The Mohave tui chub, Pahrump poolfish and White Sands pupfish all evolved in the absence of any other fishes, at least since the end of the Pleistocene, whereas Amargosa pupfish historically has co-occurred with Amargosa speckled dace (Rhynichthys osculus ssp.). Thus, desert fish species may vary in their vulnerability to impacts by western mosquitofish. Such impacts are often associated with mosquitofish predation on larvae and eggs of native fishes. Therefore, differences in antipredator behaviors may vary among desert fish species.

1.4. Chemical Alarm Cues and Epithelial Club Cells

Anti-predator traits of particular interest involve the chemical alarm cues associated with injury and their apparent detection by conspecifics (Chivers and Smith 1998; Chivers et al. 2007; Ferrari et al. 2010). Earlier studies have shown that responses to these cues can be species-specific and age-specific (Chivers and Smith 1998; Alemadi and Wisenden 2002; Lehtiniemi 2005; Olson et al. 2012). These types of cues, which are defined as signals benefiting the receiver and not the sender, have been widely observed in Ostariophysian fishes. Once injured,

epithelial tissue releases chemical cues involuntarily, alerting conspecifics of an actively foraging predator (Wisenden 2015). Detection and response to these cues allows for individual evasion and increased chances of survival. Not responding results in higher mortality rates (Wisenden 2015). Examples of responses range from a decrease in movement and hiding behavior of fathead minnows (Pimephales promelas) to area avoidance and shoaling behaviors in young convict cichlids (Amatitlania siquia) (Alemadi and Wisenden 2002; Wisenden 2015). Prey respond by reduced feeding behavior or by moving out of the water column and decreasing activity (Wisenden 2015), which in turn increased survivorship (Hews and Blaustein 1985; Chivers and Smith 1998; Wisenden et al. 1999). These antipredator reactions can be costly in terms of lost opportunities for foraging and/or breeding, especially in an ecosystem having limited resources.

Antipredator behaviors and chemical cues are also associated with club cells, specialized cells found in epithelial tissue covering the scales. Club cells may contain the chemical alarm cue, which is released when club cells are ruptured due to injury of the epidermis. Although their biological function for an individual is poorly understood, club cells may boost cell proliferation to increase protection from ultra-violet radiation and attacks by skin-penetrating parasites (Chivers et al. 2007). For example, experimental exposure to trematode cercariae induced proliferation of club cells in fathead minnows (Chivers et al. 2007). Although there are fitness benefits from club cells contributing to the role of skin as a barrier to foreign invaders, there is a demonstrable cost to club cell production. Fathead minnows produce club cells in proportion to food resources available (Wisenden and Smith 1998), and reduce club cell proliferation when challenged by cortisol injections (Halbgewachs et al. 2009). Further, male fathead minnows lose club cells completely when refraining from foraging during nest defense and egg care (Smith

1973; Smith 1976). In addition to serving as part of the immune system, many authors have noted that club cells are a prominent component of epidermal tissue, and surely are damaged during predatory attack. Thus, rupturing the club cells is likely to contribute to chemical alarm cues that elicit antipredator responses in many fishes (Pfeiffer 1977; Smith 1992; but see Carreau-Green 2008). Because there are metabolic and fitness costs to both production of club cells and behavioral responses to chemicals released from injured skin, loss of response to these cells, or possibly the loss of the cells themselves due to other processes, would render a species with reduced capacity for detecting and evading predators (Chivers et al. 2007; Ferrari et al. 2010).

Behavioral responses and club cell expression have been extensively researched in zebrafish, Danio rerio, a common model species. Alarm reactions for zebrafish were first documented as early as the 1980s, but have been expanded upon (Waldman 1982; Korpi and Wisenden, 2001; Wisenden et al. 2010). For instance, Barkhymer et al. (2018) demonstrated exposure of chemical alarm cues increased cortisol concentrations in zebrafish. Similarly, club cells have been recognized in zebrafish epidermis (Chang and Hwang 2011) and have been associated with chitin abundance (Tang et al. 2015), stress (Oliveira et al 2014), and even human skin disorders (Cline and Feldman 2016). Zebrafish have also been used to determine the neurological pathways used in alarm responses and may help establish the chromatography of alarm cues (Doving and Lastein 2009). Various studies of alarm cue responses and club cells in this species makes zebrafish a model for this area for research.

This thesis focuses on evaluating antipredator traits for several desert fishes. First, responses to injury-released chemical alarm cue were evaluated for two species native to the American southwest with similar life histories, the endangered Pahrump poolfish, Empetrichthys

latos, and the Amargosa pupfish, Cyprinodon nevadensis amargosae. I also evaluated alarm cue responses of zebrafish as a positive control. Secondly, prevalence and densities of club cells were evaluated for these fishes, along with three other desert fishes: Hot Creek Valley tui chub, Siphateles bicolor ssp, White sands pupfish, Cyprinodon tularosa, White river springfish, Crenichthys baileyi. For this club cell work, I also evaluated both zebrafish, Denio rerio, and fathead minnows, Pimephales promelas, as positive controls as these species are traditionally used in club cell research. Finally, distribution of prevalence and club cell densities were evaluated in a phylogentic context to evaluate the evolutionary history of club cells.

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2. CHEMICAL ALARM CUE RESPONSES AND EPIDERMAL HISTOLOGY OF TWO DESERT CYPRINODONTS

2.1. Abstract

Many desert fishes in the American southwest evolved in simple communities with limited fish predators or competitors following their post-Pleistocene isolation (Miller 1961; Meffe et al. 1983). Thus, *evolutionary naïvete* has been invoked to explain the decline and extirpation of desert fish populations following the introduction of non-native predaceous fishes, such as the western mosquitofish, *Gambusia affinis* (Meffe et al 1983; Sih et al. 2011). These out-sized impacts of invasive species on native species could result from the loss of costly antipredator behaviors, morphologies, and physiologies, such as a lack of anti-predator responses to conspecific injury-released chemical alarm cues. Here, the endangered Pahrump poolfish, *Empetrichthys latos*, and Amargosa pupfish, *Cyprinodon nevadensis amargosae*, were evaluated for behavioral responses to chemical alarm cues created with epithelial tissue from conspecifics. Epithelial tissue is the source of chemical alarm cues in other fishes, with a significant component being club cells. Neither poolfish nor pupfish responded to conspecific alarm cues when compared to a positive control (zebrafish). I also evaluated the prevalence and density of epithelial club cells typically associated with anti-predator chemical alarm cues in numerous fishes. Epithelial club cell prevalence was 24% and 11% of samples and club cell densities were 70.1 ± 27.8 and 9.6 ± 2.7 club cells / mm^2 of skin for poolfish and pupfish respectively. These values were substantially lower than values for zebrafish, a species extensively studied for behavioral, morphological, and physiological responses to chemical alarm cues (prevalence: 100%; density: 310 ± 51.3 club cells / mm^2). Thus, the two desert fishes had low prevalence and densities of club cells, and showed no evidence of behavioral responses to skin extract.

Therefore, antipredator competence mediated by conspecific alarm cues does not seem to be a component of the ecology of these desert fishes. These results may explain why desert fishes are vulnerable to impacts by non-native predaceous fishes.

2.2. Introduction

Many freshwater fishes in the North American desert are endemic to small, simple ecosystems, isolated from other species. Therefore, it is likely that fishes evolved under relaxed aquatic predation pressures (Minckley and Deacon 1968; Pister 1974; Rundel and Gibson 1996). In turn, lack of predator-associated selection pressure may have resulted in the evolutionary loss of costly anti-predator traits (Wisenden and Smith 1998; Chivers et al. 2007), and thus render such species more vulnerable to predators. Such *evolutionary naïvete* has been invoked to explain the rapid decline of many desert fishes following the introduction of non-native predatory species, such as western mosquitofish, *Gambusia affinis*, and red swamp crayfish, *Procambarus clarkii* (Miller 1961; Miller et al. 1989; Pister, 1974; Meffe et al. 1983; Sih et al. 2011). Management efforts to eradicate invasive species have had low rates of success (Minckley and Deacon 1968; Meffe 1985; Minckley 1995). Therefore, more research is needed to better understand mechanisms that have predisposed desert fishes to impacts by invasive species.

Evaluation of antipredator competence in desert fishes requires comparison to antipredator traits in non-insolated fishes. For instance, predator-prey interactions in non-isolated aquatic systems are often mediated by chemical cues such as the odor of predators and chemical alarm cues released by the skin of conspecifics damaged during predatory attack (Ferrari et al. 2010; Wisenden 2015). The source of alarm cues is the skin (Hintz et al. 2017). Epithelial club cells are a conspicuous component of the epithelium in the speciose superorder Ostariophysi, which contains orders such as the Cypriniformes (minnows), the Characiformes (characins and

tetras), and the Siluriformes (catfish). For these fishes, club cells have been hypothesized to be the source, or at least a component of alarm cues, chemical compositions that benefit the receiver and not the sender. (Smith 1992; see Chapter 3). Stereotypical antipredator behaviors have been documented in many species in response to conspecific alarm cues (see Smith 1992; Chivers and Smith 1998; Ferrari et al. 2010 for review).

Thus, the first step in assessing antipredator competence in desert fishes is to determine if desert fishes produce, detect, and respond to conspecific chemical alarm. This study focused on antipredator behaviors and epidermal histology of two desert fishes: Amargosa pupfish, *Cyprinodon nevadensis amaragosae,* and the federally endangered Pahrump poolfish, *Empetrichthys latos*. Amargosa pupfish were selected because, in contract to poolfish, they were able to produce numerous surviving juveniles when reared sympatrically with western mosquitofish (Goodchild and Stockwell 2016). Poolfish were chosen for this work because they were unable to produce surviving juveniles in the presence of non-native western mosquitofish (Goodchild and Stockwell 2016). One hypothesis to explain this difference is that poolfish evolved in allopatry since the end of the Pleistocene (Miller 1948). Amargosa pupfish evolved in sympatry with speckled dace (*Rhynichthys osculus ssp.*) (Williams et al. 1989), but were introduced to a fishless habitat in the 1940s by Robert Rush Miller. Here, I evaluated poolfish and pupfish for behavioral responses, and also the prevalence and density of epithelial club cells. As a positive control, I also evaluated zebrafish (*Danio rerio*) responses to injury-released alarm cues and documented the prevalence and density of their epithelial club cells. Zebrafish have well-documented alarm reactions to conspecific skin extract (Waldman 1982; Speedie and Gerlai 2008).

2.3. Materials and Methods

2.3.1. Behavioral Rearing

Pahrump poolfish (*Empetrichthys latos*) tested for behavioral responses were second generation lab-reared individuals originally collected from Spring Mountain Ranch in 2014 (Goodchild 2015). Amargosa pupfish (*Cyprinodon nevadensis amargosae*) used for the behavioral experiment were collected at River Springs, Inyo County, CA in June of 2017. This population was established in the 1940s by R.R. Miller when *C. n. amargosae* and *C. salinus* pupfish were introduced from the Amargosa River and Salt Creek, Death Valley, respectively (Miller and Alcorn 1945). Each species was kept separately at densities of 100 to 150 fish per 379-L plastic water tanks, which contained artificial foliage. Tanks were filtered using Marineland Emperor 400 Pro Series Power Filters® and equipped with an air stone. Zebrafish were obtained from EkkWill Waterlife Resources and housed in densities of 10 individuals per 38L tank. Fish were fed a diet of commercial flake food and supplemented with newly hatched brine shrimp nauplii twice per week. Temperature was maintained at approximately 22^0C with a photoperiod of 12 h light : 12 h dark.

2.3.2. Production of Test Cues

Alarm cue was produced for Pahrump poolfish, Amargosa pupfish, and zebrafish following protocols described by Wisenden (2011). Alarm cue was produced for each species by first euthanizing individual fish in a solution of 500mg/L of tricaine methanesulfonate (MS-222) (NDSU Institutional Animal Care and Use Committee protocols #A15072 and #18054), and secondly, filleting skin from both sides of the carcass. The fillets were laid flat on a piece of wet glass to measure skin area before transfer to a beaker of 50 mL dechlorinated tap water resting on crushed ice (Table 2.1). For each species, the combined skin from all individuals was

homogenized by a hand blender for 30 s, and further diluted with dechlorinated tap water to a final concentration of 1.0 cm^2 skin/mL (Table 2.1). Control cue was prepared with dechlorinated tap water. Both alarm and control cue solutions were aliquoted into 10-mL replicates and frozen at -18 ^{0}C.

2.3.3. Experimental Set Up and Procedure

Twelve 38-L glass aquaria fitted with glass lids were placed on metal racks under broad-spectrum fluorescent lights and maintained on a photoperiod of 12 h light : 12 h dark photoperiod. Each tank contained an air-powered sponge filter with an addition 2.5 m length of airline tubing inserted into the outflow of the filter to serve as a way to deliver test cues surreptitiously (Fig. 2.1). The delivery tube was secured to the metal rack to prevent movement during trials and allowed to hang below the level of the rack, out of view from the test subjects (Fig. 2.1). A grid of 5x5 cm cells was drawn on the outside of the front panel of each test tank (Fig 2.1). Black plastic was placed between tanks to eliminate social influence of fish by adjacent tanks. No viewing screens were used as these species did not show overt response to human presence and the length of the delivery tubes allowed for remote injections of test cues without test subject-human interactions.

Each test fish was transferred from rearing tanks to holding tanks within the experimental room a minimum of one week before trials began. Each fish was acclimated for 24 h in an experimental tank and randomly assigned as either alarm cue or control. Zebrafish required two individuals per trial tank to achieve pre-stimulus behavior for testing (Barkhymer et al 2018). Experimental fish were fed commercial flake food 60-75 min before trials began. For each trial, 50 mL of tank water was withdrawn from the hanging end of the delivery tube with a 60 mL syringe and discarded to remove possible contaminants from the delivery tube. An additional 50

mL of tank water was drawn and retained to be used later to flush test stimuli from the delivery tube. A Samsung camcorder mounted on level platforms was placed 1.0 to 1.5 m directly across from each test tank. The camcorder recorded events during the 5-min pre-stimulus observation period. Once completed, either control water or conspecific chemical alarm cue was introduced to the tank through the delivery tube, followed by the 50 mL flush of the previously-retained tank water. Immediately after injection of stimulus, the camcorder recorded events during the 5-min post-stimulus observation period. Following the trial, fish were measured for total length (cm) and weight (g) before placement into designated recovery tanks. Water from experimental tanks was replaced with fresh water and delivery tubes were removed and replaced with new tubes.

2.3.4. Behavioral Data Collection

Behavioral measures were vertical distribution and activity of the subject, following standard operating protocols by Wisenden (2011). Vertical distribution was determined as the vertical location of trial fish every 15 s, averaged over each 5-min observational period. Vertical distribution was determined relative to the penciled grid on the front of the test aquarium (Fig. 2.1). Using video recordings, activity was measured as the number of lines crossed by the eye of the trial fish per minute during the 5-min observational period. Data for the two zebrafish were averaged for the two fish per tank per trial. Post-stimulus data were recorded for each minute after treatment type introduction and averaged. For each trial, post-stimulus response data were analyzed using ANCOVA in JMP software (type III sums of squares, 0.05 alpha level). Treatment type (control water or alarm cue) was treated as the categorical predictor, with the pre-stimulus behavior for each fish as a covariate. I conducted 80, 80, and 50 trials for poolfish, pupfish, and zebrafish respectively.

2.3.5. Sample Preparation for Histological Examination

Fish were sacrificed using a lethal dosage of MS-222 (~500 mg/L) and a 3-4mm section of skin was taken from the nape region (Wisenden and Smith 1998; Chivers et al 2007). I sampled similar numbers of Pahrump poolfish (n = 29) and Amargosa pupfish (n = 28), but I sampled only 10 zebrafish as the presence of club cells is well-documented for this species (Beckwith et al 2000; Rakers et al 2010).

Samples were fixed in 10% formaldehyde solution for 24 h in preparation for sectioning and staining. After setting in parafilm for 8 h, samples were sectioned, stained with periodic acid Schiff reagent and counterstained with hematoxylin (PAS-H), and mounted on slides. These slides were then digitally scanned using a MoticEasyScan® slide scanner at 40X, high definition magnification.

2.3.6. Histology Analysis

Using Image-Pro Premier®, the area of epithelial tissue was calculated with smart segment tool and the number of visible club cells recorded for each sample. These data were used to estimate club cell density per mm^2 of skin per fish. An ANOVA was performed to compare club cell densities among the three species, followed by post-hoc pair-wise tests while experiment-wise alpha was maintained at 0.05.

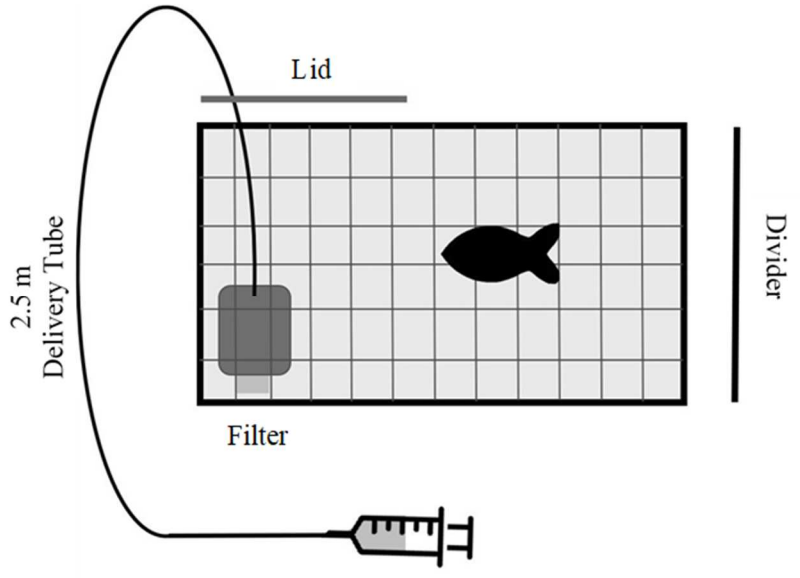

Figure 2.1. Features of behavioral trial tank. A 5 x 5cm grid was drawn on one side of a 38L glass aquarium and placed on a metal rack. A sponge filter for aeration was placed on the left-hand side of the tank. A black divider was attached to the outside between each tank. A 2.5 m delivery tube was inserted into the top of the sponge filter, attached to either the tank itself or the metal rack, and allowed to hang loose. Treatments were injected via syringes into the delivery tube.

2.4. Results

2.4.1. Behavioral Results

Zebrafish: There were significant effects of cue type ($F_{1,47} = 33.84$, $p < 0.001$; Fig. 2.2a) and pre-stimulus position ($F_{1,47} = 14.30$, $p < 0.001$; Fig. 2.2a) on vertical distribution. The post-stimulus vertical distribution was decreased from the alarm cue treatment compared to the control (Fig. 2.2a). However, zebrafish activity was not affected by cue type ($F_{1,47} = 0.444$, $p = 0.503$; Fig. 2.3a) but was significantly affected by pre-stimulus activity ($F_{1,47} = 12.395$, $p = 0.001$; Fig. 2.3a)

Poolfish: Poolfish post-stimulus vertical distribution was not significantly affected by cue type ($F_{1,69} < 0.001$, $p = 0.998$; Fig. 2.2b), but was affected by pre-stimulus position ($F_{1,64} = 100.53$, $p < 0.001$; Fig. 2.2b). Post-stimulus activity was not significantly impacted by cue type

($F_{1,64} = 1.154$, $p = 0.287$; Fig. 2.3b), but was significantly affected by pre-stimulus activity ($F_{1,69} = 70.574$, $p < 0.001$; Fig. 2.3b).

Pupfish: Pupfish post-stimulus vertical distribution was not significantly affected by cue type ($F_{1,77} = 0.90$, $p = 0.347$; Fig. 2.2c) or by pre-stimulus position ($F_{1,77} = 0.16$, $p = 0.693$; Fig. 2.2c). Post-stimulus activity was not significantly impacted by cue type ($F_{1,77} = 0.69$, $p = 0.07$; Fig. 2.3c), but was significantly affected by pre-stimulus activity ($F_{1,77} = 96.6$, $p < 0.001$; Fig. 2.3c).

2.4.2. Histology Results

Epithelial club cells were recognized as unstained, circular cells that did not open to the surface of the skin (Chivers et al. 2007). For individuals with at least one club cell, prevalence was 100% of zebrafish, 24% of poolfish, and 11% of pupfish specimens (Fig. 2.4). The estimated mean number of club cells per mm^2 of skin for zebrafish was 310 ± 51.3 (mean ± 1 SE; Fig 2.5). By contrast, poolfish and pupfish had substantially lower mean club cell densities of 17.12 ± 7.4 and 1.0 ± 0.6 club cells / mm^2, respectively (Fig. 2.5). The overall density of club cells differed significantly among all three species ($F_{2,64} = 83.8$, $p < 0.001$), with zebrafish being significantly different from both pupfish and poolfish ($F_{1,36} = 107.18$, $p < 0.001$; $F_{1,37} = 79.99$, $p < 0.001$). Pupfish and poolfish were not statistically different ($F_{1,55} = 3.41$, $p = 0.07$).

Table 2.1. Total number of individuals sacrificed, mean total length, area of skin used for each species, final solution volume, and final concentration of alarm cue are provided.

SPECIES	N	TOTAL LENGTH (mm)	SKIN AREA (cm^2)	FINAL VOLUME (ml)	CONCENTRATION (cm^2/mm)
Pahrump Poolfish	16	30.6 ± 2.13	44.11	440	1.003
Amargosa Pupfish	15	40.9 ± 4.97	53.06	530	1.001
Zebrafish	16	30.4 ± 1.53	39.69	390	1.02

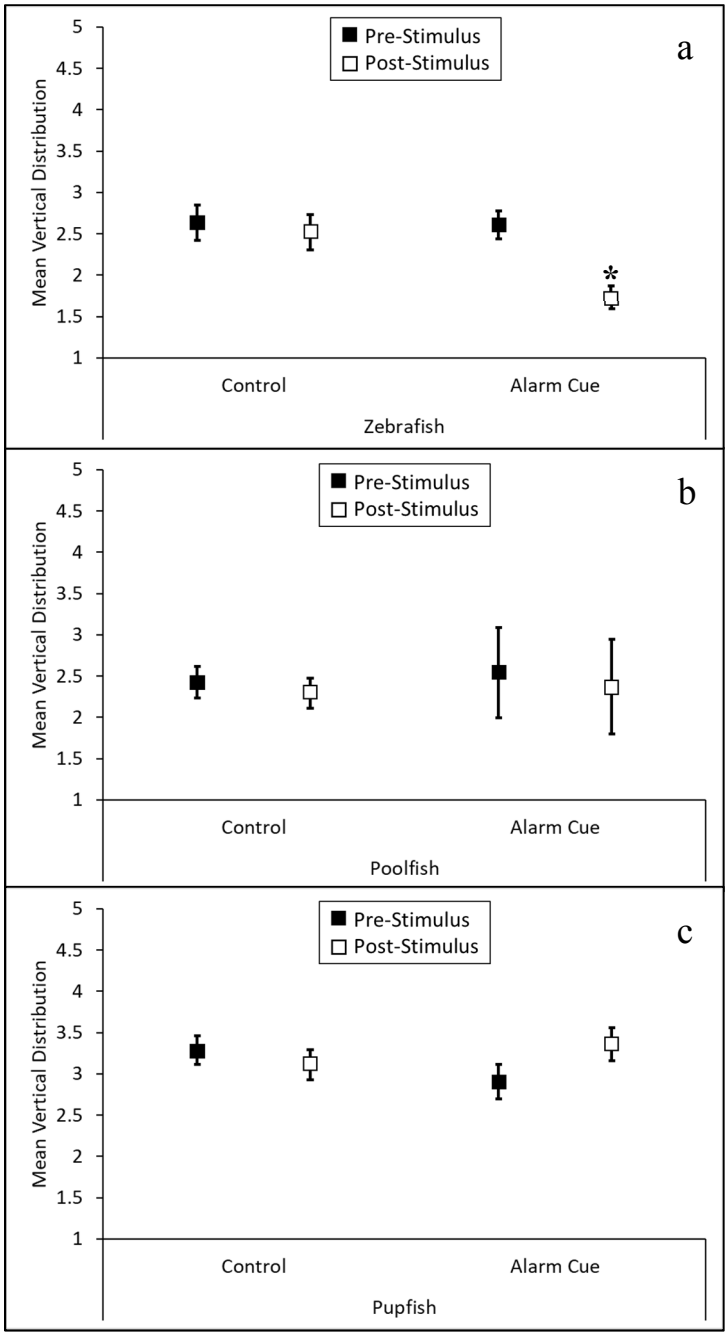

Figure 2.2. Mean ± 1 SE vertical distribution (1 = bottom, 5 = top) between treatment types (control, alarm cue) for zebrafish (a), poolfish (b), and pupfish (c) before (black square) and after (white square) introduction of test stimuli. Asterisk (*) indicates significant differences ($p < 0.05$).

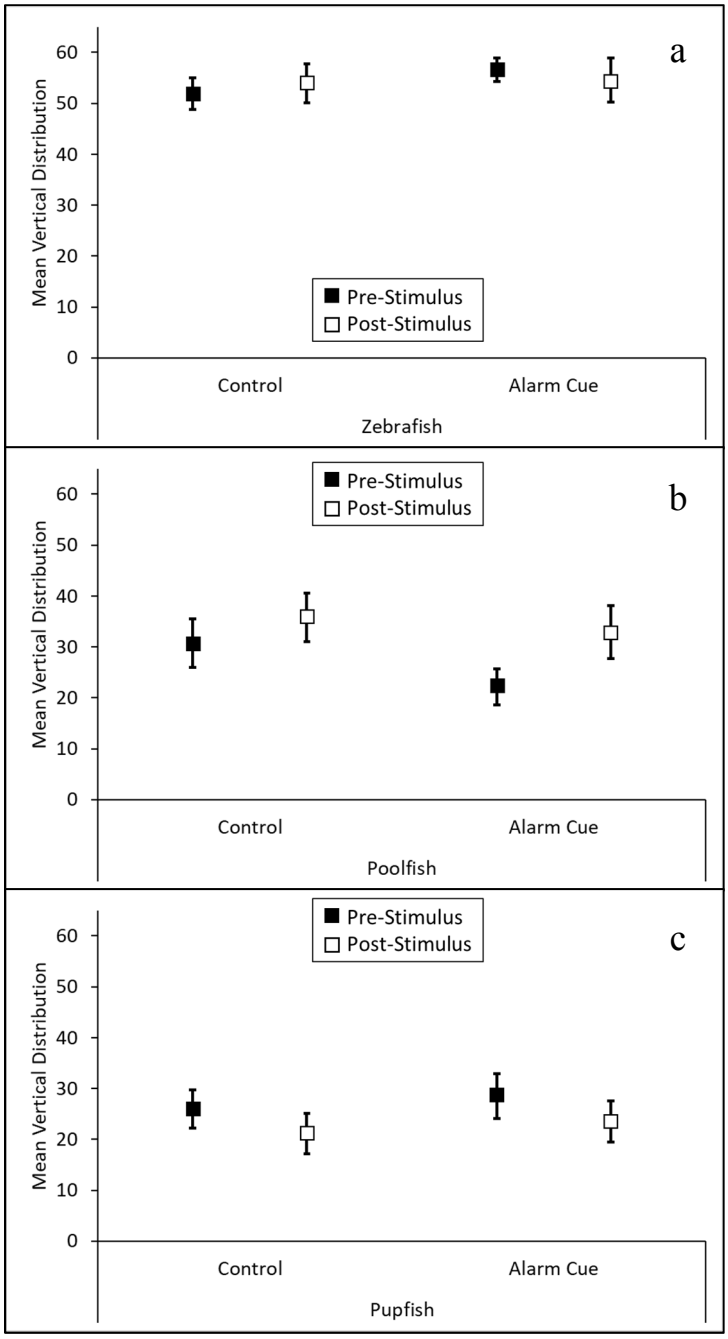

Figure 2.3. Mean ± 1 SE activity (lines crosses per min) before (black square) and after (white square) introduction of test stimuli for zebrafish (a), poolfish (b), and pupfish (c).

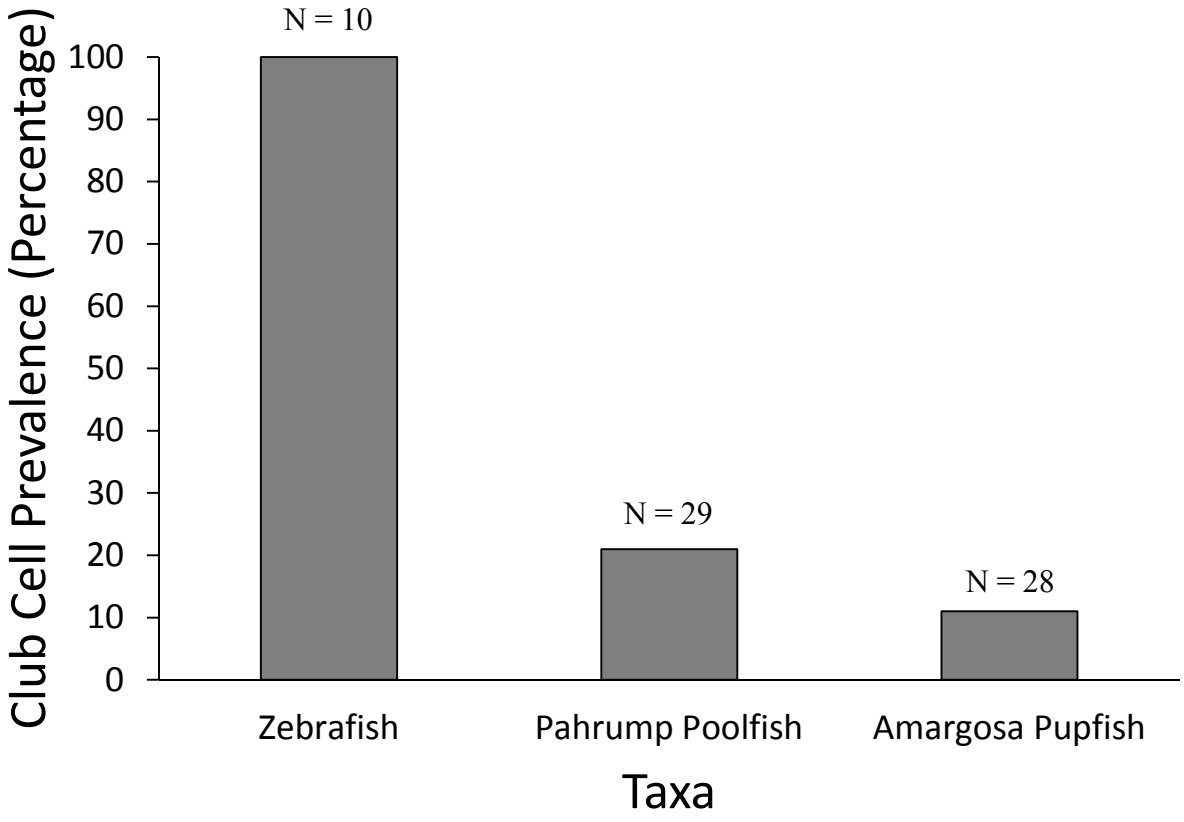

Figure 2.4. Club cell prevalence by species. A prevalence of 100% indicates that all samples displayed at least one club cells, whereas 0% indicates that none of the samples of that species exhibited club cells.

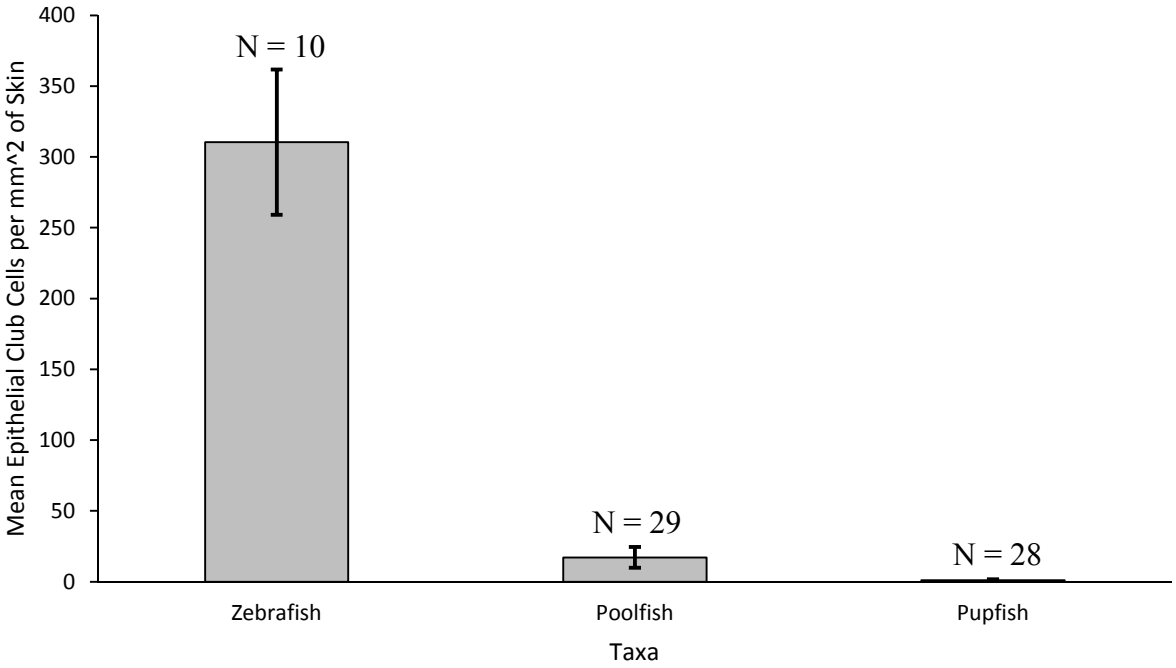

Figure 2.5. Mean ± 1 SE club cell densities among taxa. Number of samples (N) indicated above each bar.

2.5. Discussion

Similar to Chang and Hwang 2011, Zebrafish possessed numerous epithelial club cells. Their behavioral responses to chemical alarm cues in terms of changes in vertical distribution were also consistent with established literature (Barkhymer et al. 2018). By contrast, their activity unexpectedly did not change in response to alarm cue. The stereotypical response of zebrafish to alarm cues is overall reduction in activity and movement to the bottom (Ferrari et al. 2010). However, fathead minnows typically reduce activity, while zebrafish typically increase (Olson et al. 2012; Speedie and Gerlai 2008; Barkhymer et al 2018). This variation in response within a species may derived from individual personality, such that a bolder fish and shyer fish may respond differently to the same stimulus (Magnhagen and Bunnefeld 2009; Nakayama et al. 2012). Nevertheless, the change in vertical distribution exhibited by zebrafish under these

experimental conditions provides a positive control for the testing procedure used for poolfish and pupfish.

Neither poolfish nor pupfish showed any evidence of antipredator behavior in response to conspecific injury-released chemical alarm cue, consistent with the predator naïvete hypothesis. These desert fishes may have evolved reduced alarm cue responses due to relaxed predation pressure as has been observed for other isolated species (Roemer et al. 2002; Berger et al. 2007). Alternatively, non-response could be due to a limited alarm cue signal, associated with the limited club cells in both species. In either case, these desert fishes did not respond to chemical alarm cues during this study, and therefore, lack an antipredator mechanism commonly seen in many small fishes such as zebrafish.

Poolfish and pupfish had low prevalence and density of club cells relative to zebrafish. Similar club cell counts occurred in other cyprinodontids such as White Sands pupfish, *Cyprinodon tularosa*, and White River springfish, *Crenichthys baileyi* (chapter 3). In fathead minnows, club cell proliferation increases in response to skin-penetrating parasites or to acute immune challenge, increases in food, and decreases in males during breeding seasons when fasting and energetic demands for nest defense are high (Smith 1973; Wisenden and Smith 1998; Chivers et al. 2007; Halbgewachs et al. 2009). Thus, I hypothesized that the low density of club cells in cyprinodonts is likely driven by phylogenetic inertia (chapter 3), but may also stem from a history of limited exposure to parasites or extreme food restrictions. This is the first study to evaluate club cells and alarm-cue responses in desert fishes.

Collectively, this study suggests that poolfish and pupfish did not produce, detect, or react to conspecific chemical alarm cues. These results are consistent with previous research on poolfish suggesting high vulnerability to predatory fishes due to the poor juvenile recruitment

(Goodchild and Stockwell 2016), likely resulting from a history of isolation since the Pleistocene. However, Amargosa pupfish differ from poolfish in being both able to co-exist with mosquitofish (Goodchild and Stockwell 2016) and having an evolutionary history of sympatry with native Amargosa speckled dace. However, the pupfish used here were collected from an introduced population at River Springs, a habitat without other fishes.

These findings provide insight into a potential mechanism for the impact of invasive species on Pahrump poolfish and other insolated species. In fact, the lack of behavioral response to chemical alarm cue may have contributed to the decline of poolfish at Spring Mountain Ranch in 2016, where the poolfish population plummeted from well over 10,000 fish to just a few hundred fish within one year of the discovery of western mosquitofish (Kevin Guadalupe, Nevada Department of Wildlife, pers. comm). The impacts of invasive mosquitofish on Pahrump poolfish have been consistently demonstrated in experimental communities, where poolfish failed to produce juveniles when sympatric with western mosquitofish (Goodchild 2015; Goodchild and Stockwell 2016; Paulson and Stockwell, unpublished). The current study, along with evidence of population declines, are consistent with hypothesis that extended isolation in allopathy has rendered Pahrump poolfish vulnerable to invasive species due to limited antipredator behaviors.

The relevance of these results is not quite as straightforward when considering the Amargosa pupfish. The River Springs population were established before gambusia were present, but had co-evolved with Amargosa speckled dace. Further, Goodchild and Stockwell (2016) showed that pupfish were able to co-persist with mosquitofish and poolfish, with high levels of juvenile recruitment. However, pupfish do not respond to alarm cue, we must look to another mechanism to explain their co-persistence with invasive mosquitofish

Work with fathead minnows suggests the possibility that predator-naïve populations can learn to recognize invasive predators through associative recognition learning (Suboski 1990; Suboski et al. 1990; Ferrari et al. 2010). Such learning is a type of associative learning in which detection of alarm cues simultaneously with another novel, neutral stimulus, transfers the innate alarm cue responses to the novel stimulus. For fathead minnows in piscivore-free populations, recognition learning of alarm cues presented with novel predator odor, such as that of northern pike (*Esox* Lucius), allowed for the entire population to recognize the predator species in less than two weeks (Chivers and Smith 1994). Unfortunately for desert fishes, an innate behavioral response to skin extract is a pre-requisite for acquired predator-recognition of invasive predators. If desert fishes do not respond to alarm cues, the ability to use recognition learning to a non-native predator odor on desert fishes is not probable.

In conclusion, isolated poolfish and pupfish do not produce, detect, or react to chemical alarm cues. Without these behavioral responses, they are highly susceptible to predation by non-native invasive species. However, other desert species need to be evaluated to determine generality of my findings. Unfortunately, desert fish conservation will remain dependent upon human intervention to manage or remove invasive species as desert fishes may not be able to defend themselves.

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3. PREVALENCE AND DENSITY OF EPIDERMAL CLUB CELLS IN ISOLATED FISHES

3.1. Abstract

Species that evolve in isolated habitats with limited predation often have reduced investment in antipredator traits, such as the production, detection, and response to indicators of predation risk (Roemer et al. 2002; Berger et al. 2007). In aquatic ecosystems, predation risk is often mediated by so called chemical alarm cues released by tissues damaged by an attack by a predator (Smith 1992; Wisenden 2015). Epidermal tissue in many fishes contains club cells that are ruptured during an attack. The contents of these cells may contribute to the chemical component of alarm cues (Smith 1992). This study investigated whether fishes from isolated populations possess epithelial club cells. As a baseline, I evaluated two minnow species known to possess club cells, fathead minnows (Cyprinidae: *Pimephales promelas*) and zebrafish (Cyprinidae: *Danio rerio*), and five species of isolated fishes; desert-spring-dwelling Hot Creek Valley tui chub (Cyprinidae: *Siphateles bicolor ssp.*), Pahrump poolfish (Goodidae: *Empetrichthys latos*), White River springfish (Goodidae: *Crenichthys baileyi*), Amargosa pupfish (Cyprinodontidae: *Cyprinodon nevadensis*), and White Sands pupfish (Cyprinodontidae: *Cyprinodon tularosa*). The prevalence of club cells for Cyprinids (including tui chubs) ranged from 90 – 100% of individuals, whereas among Cyprinodontiformes (poolfish, pupfishes, and springfish) club cell prevalence ranged from 0 – 21%. Club cell densities per mm^2 of epithelial tissue differed significantly among species ($X^2 = 91.81$, df = 6, $p < 0.001$), pairwise comparisons revealed no differences among Cyprinids or among Cyprinodontiformes. Fathead minnows and zebrafish displayed relatively high densities of club cells per mm^2 of epithelial tissue (446.5 ± 100.3 club cells / mm^2 epithelial tissue; 310.4 ± 51.3 club cells / mm^2 epithelial tissue; mean ±

SE, respectively). Hot Creek Valley tui chub, a desert cyprinid, displayed club cell densities similar to non-isolated cyprinids sampled (137.9 ± 56.3 club cells / mm^2 epithelial tissue). In contrast, mean club cell densities were low for Pahrump poolfish (17.1 ± 8.5 club cells / mm^2 epithelial tissue), White Sands pupfish (17.1 ± 9.0 club cells / mm^2 epithelial tissue), Amargosa pupfish (1.0 ± 0.6 club cells / mm^2 epithelial tissue), and completely absent in White River springfish. Phylogeny, rather than predation history, appears to be the best predictor for expression of club cells in isolated fishes.

3.2. Introduction

Schreckstoff, or "scary stuff", refers to chemical alarm cues released during a predator attack that invoke antipredator behavioral responses in conspecifics (von Frisch 1938; Pfeiffer 1962; Stensmyr and Maderspacher 2012; Meuthen et al 2016) and has been considered a defining characteristic of ostariophysan fishes (e.g. minnows, characins, and catfishes) (Pfeiffer 1977; Nelson et al. 2016). For many fishes, the presumptive source of such "alarm cues" resides in the epidermis (Hintz et al. 2017), which is typically damaged in a predatory attack. Club cells, specialized cells found in the epidermis, have been hypothesized to contribute at least a component of alarm cues (Kristensen and Closs 2004). Although club cells are often linked with schreckstoff, understanding sender benefits and how they evolved have been the main foci of attention (Smith 1992; Chivers et al. 2007).

While schreckstoff can benefit the receiver, the benefits for the sender are less clear. There are numerous hypotheses regarding the evolution of alarm cue production, with popular hypotheses being kin selection, predator confusion, and protection from UV and parasites (Smith 1992; Chivers et al 2007). Producing alarm cues to alert shoal mates would allow closely related kin to avoid predation (Smith 1992). However, Meuthen et al. (2016) determined that relatedness

to the sender fish of nearby individuals did not significantly change their overall reaction. Smith (1992) suggested that, in the event of injury, release of chemical alarm cues may confuse predators, allowing for individuals to evade further harm. Chivers et al (2007) correlated club cell density to parasite or pathogens load, as well as exposure to UVB radiation in fathead minnows (*Pimephales promelas*). An additional study confirmed a link between club cells and immune functions, such as cortisol concentrations (Halbgewachs et al. 2009; Barkhymer et al. 2018). Therefore, club cells likely provide benefits to senders that occurred prior to their incidental role as potential sources of chemical alarm cues. Nevertheless, the presence of large cells in the epidermis provides an opportunity for receivers to develop specialized receptors to detect the contents of these cells once released in the water, and therefore, have a mechanism to detect predation risk (Dodson et al. 1994; Doving and Lastein 2009). If isolated fishes do harbor these unique epithelial club cells, then predation pressure (or lack thereof) may select for appropriate behavioral responses against predation.

Evolution of schreckstoff is the result of receiver-side selection because individuals that detect and respond to alarm cues avoid predation and, thus, have higher fitness than individuals that do not detect and respond to alarm cues (Mathis and Smith 1993; Chivers et al. 2002). Alarm responses by many fishes have been documented (Chivers and Smith 1998; Ferrari et al. 2010), but two desert fishes did not respond to alarm cues (Chapter 2). This begged the question as to whether alarm cue production was limited in these two desert fishes. However, fishes with limited predation have not yet been examined for the presence of epithelial club cells, possibly due to rarity of this phenomenon.

Desert fishes found in the American southwest evolved in allopatric populations with limited predation pressure by other fishes for upwards of 10,000 years (Soltz and Naiman, 1978;

Minckley and Deacon, 1968; Pister 1974). Enormous population declines and extirpation of various desert fishes coincided with numerous introductions of predatory invasive species, such as western mosquitofish (*Gambusia* affinis) and red swamp crayfish (*Procambarus clarkii*) (Miller 1961; Miller et al. 1989; Minckley and Deacon 1968; Cox and Lima 2006; Sih et al. 2011). One suggestion for the vulnerability of isolated species to invasive species is the hypothesized loss of antipredator behaviors due to relaxed predation pressures. I previously tested two desert fishes for behavioral responses to alarm cue substances and found none (chapter 2). Here, we extend that study to evaluate prevalence and densities of epithelial club cells among select desert fishes, comparing these measures to those of two well-studied cyprinid species.

Outside of the Ostariophysian fishes, little is known about club cells. The aim of this study was to determine the status of club cells in several taxa of desert fishes; four species in the order Cyprinodontiformes: Pahrump poolfish (*Empetrichthys latos*), Amargosa pupfish (*Cyprinodon nevadensis amargosae*), White Sands pupfish (*Cyprinodon tularosa*), and White River springfish (*Crenichthys baileyi*). There was one desert fish species in the Cypriniformes: Hot Creek Valley tui chub (*Siphateles bicolor ssp.*). To provide a baseline for this study, I also sampled two additional members of the Cypriniformes, fathead minnows (*Pimephales promelas*) and zebrafish (*Danio rerio*) as these two species are known to possess both club cells and behavioral responses to club cell containing skin extracts (Smith 1992; Wisenden and Smith 1998; Chivers et al. 2007). The limited alarm reactions to skin-extract by the two desert fishes examined in Chapter 2 led me to predict that these desert fishes would have low club cell prevalence and densities relative to fathead minnows and zebrafish. To make this comparison, I standardized a protocol for quantifying club cells in fish skin.

3.3. Materials and Methods

3.3.1. Population Sources

Pahrump poolfish were obtained from two populations: Spring Mountain Ranch near Las Vegas, NV (36°04'16.9"N 115°27'13.7"W) in 2014 and Shoshone Spring near Ely, NV (38°56'21.8"N 114°25'04.6"W) in 2017. White River springfish were descended from fish collected at Moapa Warm Springs near Moapa, NV (36°44'12.2"N 114°44'20.5"W) in 2012. Amargosa pupfish were collected in 2017 from a non-native population at River Springs (Mono County; 37^0 56' 15.0" N; 118^0 36' 44.8" W) This population was established in 1940 by Robert Rush Miller using fish from two pupfish species; 350 *C. n. amargosae* from the Amargosa River and 425 *C. salinus* from Salt Creek, Death Valley National Monument (Miller 1968). The River Springs population was genetically descended from *C. n. amargosae* (Steve Parmenter, California Department of Fish and Game, pers. Comm.). Hot Creek Valley tui chubs were collected in 2017 near Tonopah, Nye County, NV (32°11'03.8"N 116°09'06.9"W).

White Sands pupfish were collected and preserved in 2006 from an experimental pond on Holloman Air Force Base, Otero County, NM (32°49'25.2"N 106°06'46.6"W). This population was established in 2001 using 200 fish from native Salt Creek population of *C. tularosa* (Collyer et al. 2007). Fathead minnows were obtained from Scheels® Sporting Goods in Fargo, ND. Zebrafish were acquired from EkkWill Waterlife Resources in Ruskin, FL.

3.3.2. Sample Preparation

Fish were sacrificed using a lethal doseage of MS-222 (~500 mg/L; IACUC protocol #A15072) and a 3-4mm section of skin was taken from the nape. Sample sizes for each species varied from 6 to 30 individuals; Pahrump poolfish (n = 29), Amargosa pupfish (n = 28), White

Sands pupfish (n = 30), and White River springfish (n = 10), zebrafish (n = 10), fathead minnows (n = 10), and Hot Creek Valley tui chubs (n = 6).

Histological preparation followed protocols previously used by Chivers et al (2007). Briefly, samples were fixed in 10% formalin solution for 24 h in preparation for sectioning and staining. After setting in parafilm for 8 h, samples were stained with periodic acid Schiff reagent and counterstained with hematoxylin (PAS-H). Samples were then thin sliced and mounted on slides. These slides were digitally scanned using a MoticEasyScan slide scanner at 40X, high definition magnification.

3.3.3. Statistical Analyses

Prevalence of samples displaying at least one club cell were tested using chi-square contingency tables to test for i) species x club cell presence, ii) order x club cell presence. Using Image-Pro Premier®, the area of epithelial tissue was calculated with smart segment digital tool and the number of visible club cells recorded for each sample. These data were used to estimate club cell density per mm^2 of skin per fish. We tested for inter-specific difference in club cell densities by including only individuals with at least one club cell.

3.4. Results

3.4.1. Prevalence

Club cell prevalence varied widely among species, from 0% to 100% ($X^2_{1,6} = 57.7$, $p < 0.001$). This variation was largely driven by high variation between orders ($X^2_{1,1} = 52.3$, $p < 0.001$), with high prevalence for cyprinids (96.7% ± 3.3%) and low prevalence for cyprinodontids (16.3% ± 7.0%). Furthermore, there was nonsignificant variation within both cyprinids and cyprinodontids ($X^2_{1,2} = 1.7$, $p > 0.05$; $x^2_{1,3} = 7.5$, $p > 0.05$, respectively). Club cells were observed in 90% of fathead minnows (n = 10) 100% of zebrafish (n = 10), and 100% of

Hot Creek Valley tui chub (n = 6) (Table 3.1; Fig. 3.1). By contrast, prevalence for the four cyprinodontiformes was notably lower: 33% of White Sands pupfish (n = 30), 21% of Pahrump poolfish (n = 29), 11% of Amargosa pupfish (n = 28), and 0% of White River springfish sampled (n = 10) (Table 3.1; Fig. 3.1).

3.4.2. Densities

Mean club cell densities (estimated number of club cells per mm^2 of skin) differed significantly among species ($F_{6,48} = 13.30$, $p < 0.001$) (Table 3.1). Hot Creek Valley tui chubs (313.7 ± 56.3, mean ± SE), zebrafish (310.4 ± 51.3), and fathead minnows (446.5 ± 100.3) were not statistically different ($F_{2,22} = 2.12$, $p = 0.143$) (Fig. 3.2). Similarly, club cell densities were not significantly different among the four Cyprinodontiformes sampled: Amargosa pupfish (1.03 ± 0.62), White Sands pupfish (17.1 ± 8.9), Pahrump poolfish (17.12 ± 7.4), and White River springfish (0.0 ± 0.0) ($F_{3,25} = 2.18$, $p = 0.115$) (Fig. 3.2). In general, fishes in the Cyprinodontiformes order had significantly lower densities than those in the Cypriniformes order ($F_{1,53} = 64.43$, $p < 0.001$). Notably, Hot Creek Valley tui chub had significantly more club cells than the other desert fishes ($F_{4,31} = 17.85$, $p < 0.001$) (Fig. 3.2).

Table 3.1**.** Number of samples (N), prevalence (%), and densities (mean ± SE club cells / mm^2 of skin) per species.

COMMON NAME	**SPECIES**	**ORDER**	**N**	**PREVALENCE (%)**	**DENSITIES (Club cells / mm^2 epithelial tissue)**
Fathead minnows	*Pimephales promelas*	Cypriniformes	10	90	446.5 ± 100.3
Zebrafish	*Danio rerio*	Cypriniformes	10	100	310.4 ± 51.3
Hot Creek Valley tui chub	*Siphateles bicolor ssp.*	Cypriniformes	6	100	313.7 ± 56.3
White Sands pupfish	*Cyprinodon tularosa*	Cyprinodontiformes	30	33	17.1 ± 7.36
Pahrump poolfish	*Empetrichthys latos*	Cyprinodontiformes	29	21	17.1 ± 9.0
Amargosa pupfish	*Cyprinodon nevadensis amargosae*	Cyprinodontiformes	28	11	1.0 ± 0.6
White River Springfish	*Crenichthys baileyi*	Cyprinodontiformes	10	0	0.0 ± 0.0

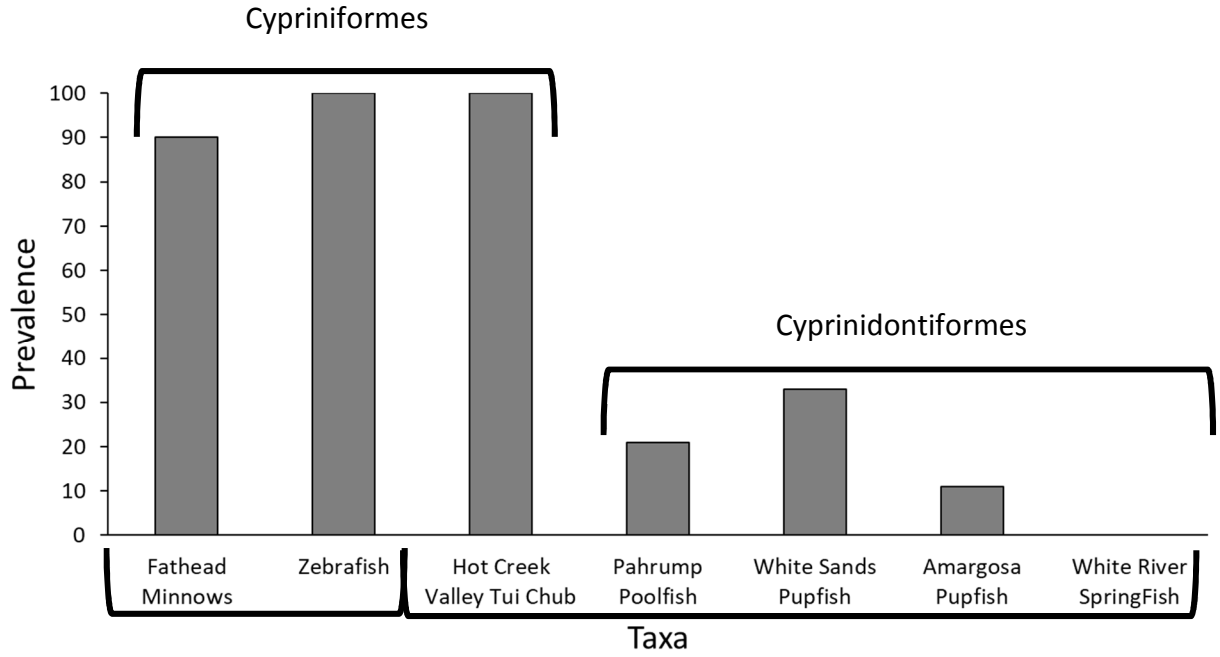

Figure 3.1. Percentage prevalence of club cells by species for sample sizes reported in Table 3.1.

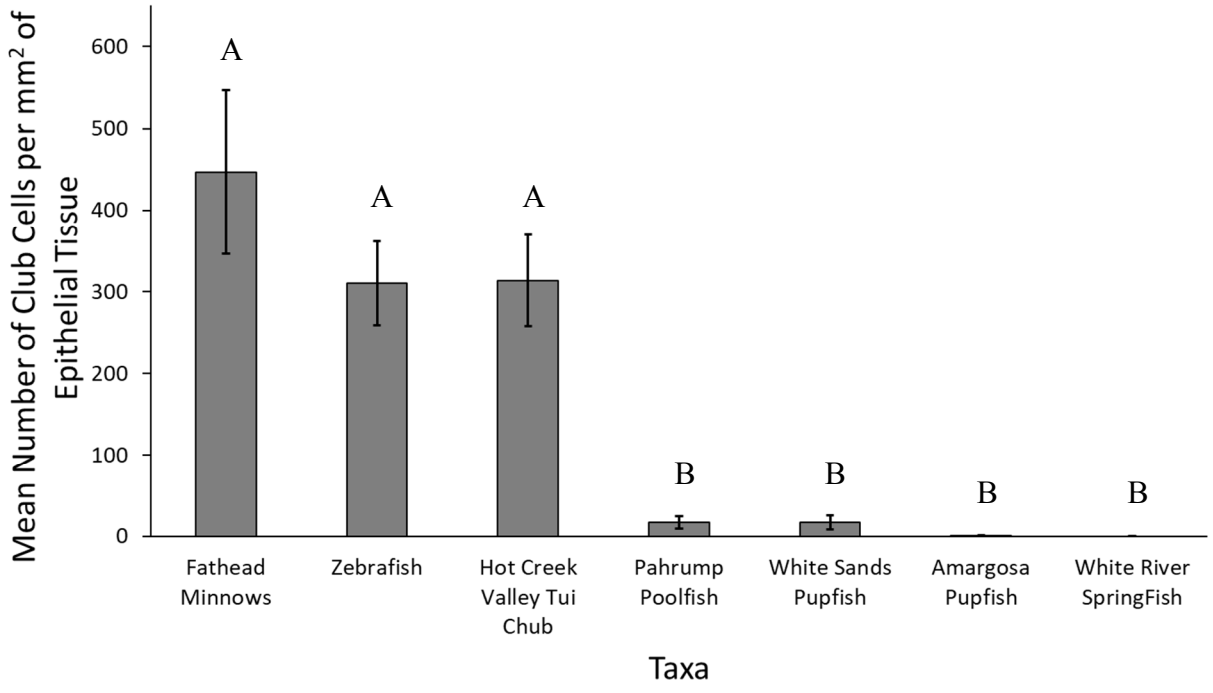

Figure 3.2. Mean ± 1 SE club cell densities (club cells / mm^2 of skin) among taxa. Letters indicate statistically similar taxa.

3.5. Discussion

Epithelial club cell presence in fathead minnows and zebrafish was consistent with previous literature, serving as a positive control for methodology and club cell identification (Chivers et al 2007; Menke et al 2011). Hot Creek Valley tui chubs, an isolated desert cyprinid, displayed prevalence and densities statistically indistinguishable from zebrafish and fathead minnows, suggesting that club cell presence is driven by phylogenetic inertia (Pfeiffer 1977; Smith 1992).

For the four cyprinodontid species, prevalence varied from 0% - 33%, which was significantly lower than for the three cyprinid species (90% - 100%). I observed club cells for the three cyprinodontids that had better sampling (n = 28 – 30), but did not observe club cells for the more modestly sampled White River springfish (n = 10). Additional sampling is warranted to verify club cell absence in White River springfish.

Densities were calculated as club cells per mm^2 of epithelial tissue, similar to the reporting format used in other studies of epithelial histology (Srivastave et al. 1988; Warren et al. 1991; Patel et al. 2015; Norte Dos Santos 2018). For the three cyprinids sampled, the mean club cell density (373.4 club cells / mm^2 of skin) was more than an order of magnitude higher than for the four cyprinodontids (32.9 club cells / mm^2). Higher density of club cells in cyprinids may increase concentration of alarm cues, resulting in the decreased vertical distribution response seen in zebrafish (Chapter 2). As stated before, club cells have been the hypothesized source for the alarm cues that elicit the responses. (Smith 1992). Low densities of club cells in cyprinodontids may decrease alarm cue concentrations, resulting in no responses to alarm cue seen in poolfish and pupfish (Chapter 2).

Both prevalence and density data suggest phylogeny may constrain club cell development. To evaluate phylogenetic patterns of club cell prevalence and densities, we surveyed the literature using the following key terms: alarm cue, club cells, epithelial, fishes, and skin. We found 31 citations that reported club cells for 40 of the 47 species, with club cells found in eight of the 11 orders evaluated (Table 3.2, Fig. 3.3).

For many studies, the number of individuals evaluated was not reported. For the seven species in the survey that lacked club cells, sampling was often modest (< 20 or unknown). For example, evaluation of club cells in five species of fish in the Characiformes found that four species had club cells (Junior et al. 2012; Alves et al. 2013; Camacho et al. 2016). However, the species reported as lacking club cells was based on a sampled size of only four individuals. Thus, club cells appear to be ubiquitous within fishes, but un-even and inadequate sampling make it difficult to detect evolutionary patterns.

Furthermore, club cell densities are not typically reported in the literature, making comparison difficult. Out of the 47 fish species evaluated, club cell densities were only reported for four species: fathead minnow (*Pimephales promelas*), johnny darter (*Etheostoma nigrum*), yellow perch (*Perca flavescens*), and North African catfish (*Clarias gariepinus*) (Guerra et al. 2006; Chivers et al. 2007). These studies reported densities as either mean number of club cells in epidermis per ocular diameter (400X) or as volumetric density of epidermis tissue (Guerra et al. 2006; Chivers et al. 2007). In contrast, I reported club cell densities as club cells / per mm^2 of skin per sample. Reporting densities as club cells / per mm^2 of skin is consistent with other epithelial tissue research, allowing for ease of cross-examination with other works.

My work has shown the importance of intensive sampling. In fact, prevalence as low as 11% were observed (Amargosa pupfish), but such species would be likely classified as lacking

club cells if they were not adequately sampled. A sample size of about 30 individuals would be a necessary threshold to confidently detect the presence of club cells when prevalence is near 10%. Our sampling has provided additional coverage, but has also demonstrated the importance of adequate sampling for assessing the presence or absence of club cells. To date, these finding suggest that club cells, rather than being a defining characteristic of Ostariophysian fishes, are broader in distribution. More intensive sampling will be necessary to evaluate if club cells have been lost in any taxa, including desert fishes such as the White River springfish.

Table 3.2. Fish orders, number of species examined (N), club cell prevalence within order (percentage of sampled species with club cells), and limited sampling (number of studies with less than 20 individuals or unreported sample sizes) reviewed from literature.

SUBCLASS and SUPERORDERS	ORDERS	N	PREVALENCE	LIMITED SAMPLING	CITATION NUMBERS
SubCl Chondrostei	Polypteriformes	1	100	0	31
SubCl Neoptergyii					
Ostariophysi	Cypriniformes	12	100	11	3, 7, 10, 16, 17, 18, 20, 21, 28, 29, This Study
Ostariophysi	Characiformes	5	75	3	1, 4, 9, 11
Ostariophysi	Siluriformes	9	100	8	3, 6, 13, 14, 21, 24, 26, 27, 30, 32
Protacanthopertygii	Salmoniformes	2	50	2	22, 25
Protacanthopertygii	Ophidiiformes	1	100	1	32
Acanthopterygii	Cyprinodoniformes	7	72	5	3, 16, This Study
Acanthopterygii	Perciformes	5	100	3	3, 5, 19, 23
Acanthopterygii	Beloniformes	1	0	1	30
Acanthopterygii	Scorpaeniformes	2	0	2	7, 8
Acanthopterygii	Tetraodontiformes	1	100	1	27

(1.Alves et al. 2013; 2.Barreto et al. 2014; 3.Bryant 1986; 4.Camacho et al. 2016; 5.Chivers et al. 2007; 6.Gurrea et al. 2006; 7.Halacka et al. 2010; 8.Halacka et al. 2012; 9.Ide et al. 2003; 10.Irving 1996; 11.Junior and Hoffman 2007; 12.Junior et al. 2010; 13.Kumari et al. 2009; 14.Lizarazo et al. 2008; 15.Mathuru 2016; 16.Mokhtar 2015; 17.Pakk et al. 2011; 18.Pollock et al. 2012; 19.Putys et al. 2015; 20.Rakers et al. 2009; 21.Ralphs and Benjamin 1992; 22.Russell et al. 2008; 23.Sanches et al 2015; 24.Smith 2000; 25.Stabell and Vegusdal 2010; 26.Shiomi et al. 1988, 27.Thomson 1969; 28.Tripathi and Mittal 2010; 29.Tripathi et al. 2008; 30.Tsutsui et al. 2011; 31.Whitear 1981; 32.Zaccone et al. 1989)

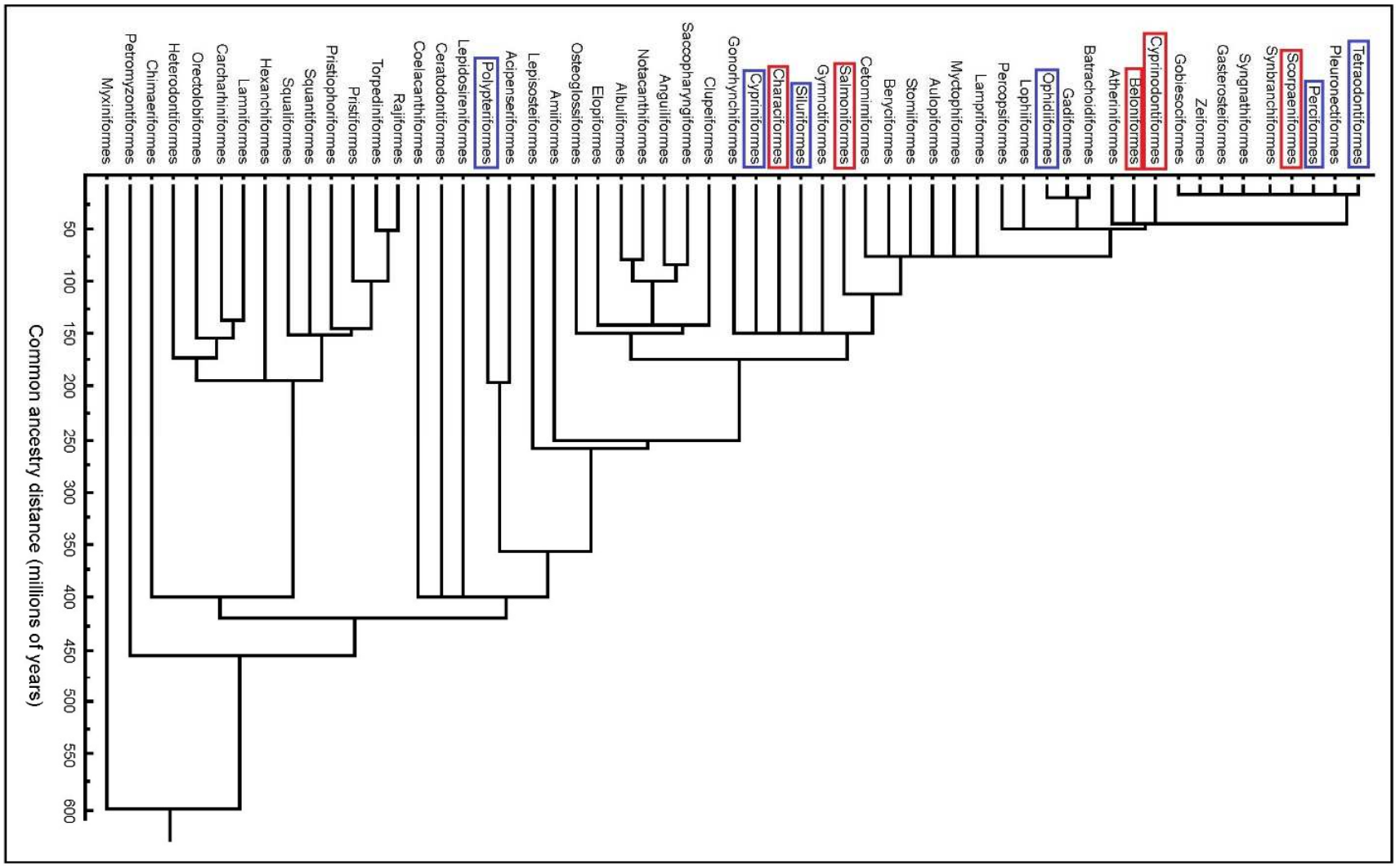

Figure 3.3. Fish phylogeny depicting orders evaluated for club cell presence. Red boxes indicate orders with less than 100% prevalence and blue boxes indicate orders with 100% prevalence.

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4. CONCLUSIONS AND FUTURE DIRECTIONS

The susceptibility of desert fishes to predatory invasive species has consequences for their survival. Loss of antipredator traits can make species more vulnerable, similar to isolated terrestrial systems and relaxed antipredator responses (Roemer et al. 2002; Berger et al. 2007). Behavioral evidence here suggests that isolated desert fishes may have lost response to injury-released chemical alarm cues, which is closely associated with predatory avoidance and survivorship. However, data on development of epithelial club cells, the hypothesized source of chemical alarm cues for other fishes, suggests phylogeny may over-ride evolutionary naïvete. Additional sampling will be informative, as species such as pupfish and tui chubs appear to be able to co-persist with invasive species whereas others such as Poeciliopsis and Pahrump poolfish have not. Unfortunately, population declines are likely without aggressive management against invasive species and monitoring of desert fishes.

Future research should broaden this research to other desert fishes in the American southwest to verify the generality of these findings. Earlier detection and plasticity of this detrimental aspect of desert fish ecology will lead to improved management tactics or even persistence of populations.

APPENDIX A. PHYSIOLOGICAL STRESS RESPONSES TO ALARM CUES IN ZEBRAFISH (DANIO RERIO)

A.1. Methods

A.1.1. Rearing

Zebrafish were obtained from EkkWill Waterlife Resources and housed in densities of 10 individuals per 38L tank. Tanks were equipped with sponge filters. Fish were fed a diet of commercial flake food and supplemented with newly hatched brine shrimp nauplii twice per week. Temperature was maintained at approximately 22^0C with a photoperiod of 12 h light : 12 h dark.

A.1.2. Experimental Set Up

Twelve 38-L glass aquaria fitted with glass lids were placed on metal racks under broad-spectrum fluorescent lights and maintained on a photoperiod of 12 h light : 12 h dark photoperiod. Each tank contained an air-powered sponge filter with an addition 2.5 m length of airline tubing inserted into the outflow of the filter to serve as a way to deliver test cues surreptitiously. The delivery tube was secured to the metal rack to prevent movement during trials and allowed to hang below the level of the rack, out of view from the test subjects. Black plastic was placed between tanks to eliminate social influence of fish by adjacent tanks. No viewing screens were used as zebrafish did not show overt response to human presence and the length of the delivery tubes allowed for remote injections of test cues without test subject-human interactions.

Zebrafish required two individuals per trial tank to achieve pre-stimulus behavior for testing (Barkhymer et al 2018). Each pair of test fish were acclimated for 24 h in an experimental

tank and randomly assigned as either alarm cue or control. Experimental fish were fed commercial flake food 60-75 min before trials began. For each trial, 50 mL of tank water were withdrawn from the hanging end of the delivery tube with a 60 mL syringe and discarded to remove possible contaminants from the delivery tube. An additional 50 mL of tank water was drawn and retained to be used later to flush test stimuli from the delivery tube. Either control water or conspecific chemical alarm cue was introduced to the tank through the delivery tube, followed by the 50 mL flush of the previously-retained tank water. After a 5-min. post-stimulus period, fish were euthanized in solution of MS-222 (~500 mg/L; IACUC protocol #A15072) and frozen in 50mL glass vials. Each aliquot represented a replicate.

A.1.3. Cortisol Extraction

Whole-body cortisol extraction was adapted from Canavello et al. (2011). Individual fish were partially thawed, weighed to the nearest 0.01mg, minced, and homogenized in 1mL of ice-cold phosphate buffered saline (PBS) using a Cole-Parmer LabGen 125 homogenizer in 5 x 15s bursts followed by homogenizer wash with an additional 2 x 1 mL PBS. The homogenate and washes were combined and extracted with 5, 3 mL volumes of diethyl ether. The resulting solution was cortex-mixed for 1 min then centrifuged at 2075 *g* for 5 min to separate aqueous and ether layers. The upper ether layers containing cortisol were removed and combined. Ether extracts were evaporated in a fume hood overnight to near dryness. Samples from individual fish were reconstituted with 1 ml PBS for 24 h. All steps were carried out at 4℃. Enzyme-linked immunosorbent assay (ELISA) for human salivary cortisol (Salimetrics; www.salimetrics.com) modified for whole body *D. rerio* was used to quantify cortisol (Canavello et al. 2011). Absorbances from reactions were measured at 450 nm using a BioTek Synergy HTX plate reader

(www.biotek.com). Net absorbances were determined by subtracting non-specific binding values. Cortisol concentrations in 0.1 ml samples were determined by comparison to BB_0^{-1} (*i.e.,* net sample A450 per zero control A450) vs cortisol (0 – 3.0 µg dl^{-1}). All samples were assayed in triplicate and resultant cortisol levels were normalized based on fish mass and expressed as ng cortisol g^{-1} fish mass.

A.2. Results

Cortisol measurements were averaged per trial tank and reported as Ln [ng g^{-1}]. A mixed model was used to analyze the data. Average cortisol concentrations were approximately 50% higher in alarm cue exposed fish (1.54 ± 0.15 Ln [ng g^{-1} fish mass]; mean ± SE) compared to control fish (0.97 ± 0.18 Ln [ng g^{-1} fish mass]) ($F_{1,48} = 5.84$, $p = 0.019$, Fig. A.1).

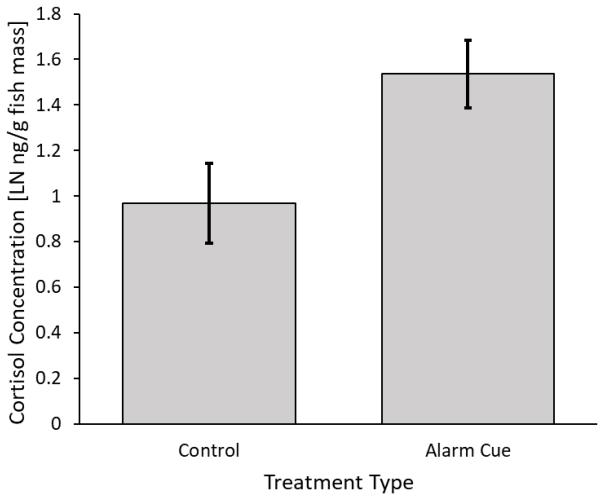

Figure A.1. Mean ± 1 SE of whole-body cortisol extractions for zebrafish in each treatment type (alarm cue vs control) are shown.

A.3. References

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APPENDIX B. CLUB CELL DENSITY COMPARISON BETWEEN WILD-TYPE AND LAB-REARED FATHEAD MINNOWS (PIMEPHALES PROMELAS)

B.1. Methods

B.1.1. Rearing

Wild-type fathead minnows were obtained from Scheels®, and caught in local water sources around Fargo, ND. Lab-reared fathead minnows were obtained from U.S. Environmental Protection Agency (EPA) in Duluth, MN. Each group were housed in sets of eight in 38L tanks with airstones for aeration. Fish were fed a diet of commercial flake food. Temperature was maintained at approximately 22^0C with a photoperiod of 12 h light : 12 h dark.

B.1.2. Sample Preparation and Analysis

Fish were sacrificed using a lethal doseage of MS-222 (~500 mg/L; IACUC protocol #A15072) and a 3-4mm section of skin was taken from the nape. Sample sizes consisted of 10 wild-type and lab-reared individuals.

Histological preparation followed protocols previously used by Chivers et al (2007). Briefly, samples were fixed in 10% formalin solution for 24 h in preparation for sectioning and staining. After setting in parafilm for 8 h, samples were stained with periodic acid Schiff reagent and counterstained with hematoxylin (PAS-H). Samples were then thin sliced and mounted on slides. These slides were digitally scanned using a MoticEasyScan slide scanner at 40X, high definition magnification. Prevalence and club cell densities (club cells/ mm^2 of epithelial tissue) were calculated. Club cell densities were analyzed using a non-parametric Mann Whitney U test.

B.2. Results

Overall prevalence, number of sampled displaying at least one club cell, was 90% for wild-type fathead minnows and 100% for lab-reared fathead minnows. Club cell density for wild-type fathead minnows (446.52 ± 100.31 club cells / mm^2 epithelial tissue; mean ± SE) was significantly lower than club cell density for lab-reared fathead minnows (839.18 ± 141.11) ($F_{1,8}$ = 5.144, p = 0.036; Fig. B.1).

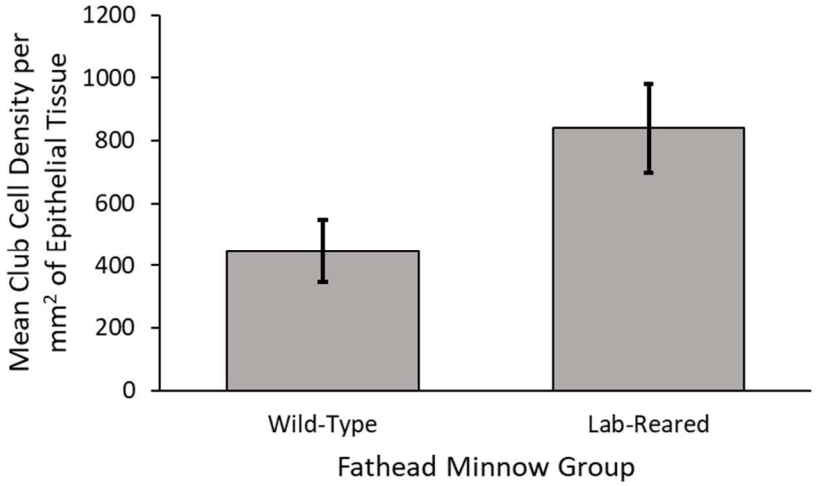

Figure B.1. Mean ± 1 SE comparison of club cell density per mm^2 of epithelial tissue between wild-type and lab-reared fathead minnows is shown.

B.3. References

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additional help.